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NR8383 Alveolar Macrophage Toxic Growth Arrest by Hydrogen Peroxide Is Associated with Induction of Growth-Arrest and DNA Damage-Inducible Genes *GADD45* and *GADD153*

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NR8383 Alveolar Macrophage Toxic Growth Arrest by Hydrogen Peroxide Is Associated with Induction of Growth-Arrest and DNA Damage-Inducible Genes *GADD45* and *GADD153.* Patton, G. W., Paciga, J. E., and Shelley, S. A. (1997). *Toxicol. Appl. Pharmacol.* **147, 126–134.**

Breathing air exposes humans and other mammals to various toxic agents including oxidative contaminants associated with fine particles of less than 2.5 μm which may be deposited in the deep lung and have been implicated in the increased morbidity and mortality correlated with air pollution. Oxidative damage from inhaled particles may include damage to DNA, thereby adversely affecting the immunosurveillance provided by alveolar macrophages. Using the rat alveolar macrophage cell line NR8383, we demonstrated that cell proliferation was inhibited by exogenous hydrogen peroxide, an oxidant naturally produced in cellular respiration and phagocytosis. Mercaptosuccinate, a specific inhibitor of the antioxidant enzyme glutathione peroxidase, also inhibited cell growth. Genes known to be coordinatively regulated in response to growth arrest and DNA damage, *GADD45* and *GADD153,* were induced compared to the housekeeping gene β-*ACTIN* by equitoxic doses of hydrogen peroxide and mercaptosuccinate. Hydrogen peroxide treatment of cells in which glutathione peroxidase was inhibited by mercaptosuccinate resulted in even greater induction of both GADD genes. This approach using the NR8383 alveolar macrophage cell line provides a model for studying genotoxicity at the mechanistic level at which stress-responsive genes involved in growth arrest and DNA-damage response are modulated. © 1997 Academic Press

Understanding the mechanisms for cytotoxicity of the tens of thousands of inhaled particles that are acquired by human lungs each day poses serious challenges due to the complex nature of the myriad types of particles found in air. Studies of toxic synergism and antagonism of metals in dusts found that even two-element combinations are too simple a tool for predicting toxic effects (Geertz *et al.,* 1994). Thus, assessing the impact of anthropogenic air pollutants on human health remains a problem. It is well understood that alveolar macrophages, the ''dust cells'' that phagocytize particles and pathogens in the lungs, have many roles including protecting against bacterial and viral infection, gathering particles for export up the mucociliary escalator, and the production of more than 100 chemokine and cytokine signals necessary for the normal function and responsiveness of mammalian lungs (Seljelid and Eskeland, 1993). Particles with toxic constituents potentially have the capacity to damage macrophages, abrogating function. Genotoxic interference with alveolar macrophage function can seriously impair both humoral and nonhumoral protection of the respiratory organs as shown by H_2O_2 inhibition of phagocytosis (Oosting *et al.,* 1990). Indeed, such a mechanism has been suggested in the etiology of the elevated incidences of cardiopulmonary disease and lung cancer seen in epidemiological studies (Dockery *et al.,* 1993; Pope *et al.,* 1995).

Inhalable airborne particulates may contain many oxidative agents including silicates, some volatile organic compounds, reactive metals and other oxidizing compounds. Also, phagocytized mineral dust particles enhance endogenous generation of free radicals (Vallyathan *et al.,* 1992). The biomolecular targets of oxidation are diverse and include proteins, lipids, carbohydrates, and nucleic acids. A classic example is the chain reaction involving peroxidation of unsaturated lipids in the absence of adequate levels of antioxidant defenses such as vitamin E and selenium-dependent glutathione peroxidase (GPx). Direct damage to DNA can be caused by cellular exposure to oxidative agents in the absence of adequate antioxidant defenses, and our interest is in the ability to detect early response to DNA damage as a biomarker for environmental oxidative stress. Since oxidation can affect every level of the cellular response pathways including receptors, transduction factors and regulatory proteins, and can cause direct oxidative damage to nucleotides, multiple pathways critical to the multitude of alveolar macrophage functions potentially could be affected and further complicate attempts to address questions of toxic mechanisms.

Our approach focuses on the central cellular response to oxidative damage sufficient to invoke cell cycle arrest, i.e., induction of the growth arrest and DNA damage-inducible (GADD) genes. GADD genes were originally identified on

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the basis of rapid induction in proliferating Chinese hamster ovary (CHO) cells by UV irradiation (Fornace *et al.*, 1988). GADD genes have been identified in human and rodent tissues and found to be induced by other types of DNA-damaging agents and some growth arrest conditions. *GADD45*, which can be induced via either p53-dependent or p53-independent pathways, codes for a 165-amino acid polypeptide involved in DNA replication and repair. DNA strand breaks are sufficient for wild-type p53 induction in response to DNA-damaging agents (Nelson and Kastan, 1994) and H_2O_2 has been shown to induce DNA strand breaks in human and other primate *in vitro* studies (Meneghini and Hoffmann, 1980) and *ex vivo* studies (Lee *et al.*, 1996). Humans exposed to urban pollution showed changes in nasal respiratory epithelium and elevated levels of single strand breaks (Calderón-Garcidueñas *et al.*, 1996), underscoring the relevance of such investigations to human and environmental health.

To understand better the changes that occur in alveolar macrophages in response to oxidative challenge from constituents of environmental contaminants, we have examined expression of *GADD45* and *GADD153* (murine: *CHOP-10*) in an alveolar macrophage cell line using the hydrogen peroxide-derived hydroxyl radical as a model of oxidant-induced injury. NR8383, a well-characterized, spontaneously transformed alveolar macrophage cell line from a male Sprague–Dawley rat, was used in this study. These cells exhibit the macrophage properties of phagocytosis, nonspecific esterase activity, Fc receptors, oxidative burst, interleukin-1 secretion, and responsiveness to exogenous growth factors (Helmke *et al.*, 1989). The ability of these cells to generate H_2O_2 following incubation with *Pneumocystis carinii* or zymosan has been demonstrated (Hidalgo *et al.*, 1992). *In vitro* cell proliferation studies and reverse-transcriptase polymerase chain reaction (RT-PCR) of RNA using NR8383 showed that doses of H_2O_2 sufficient to retard cell growth produced induction of *GADD45* and *GADD153*, suggesting that the method might provide a simple means to detect oxidative stress by exogenous agents. Also, as has been mentioned by others (Crawford *et al.*, 1996), studying the adaptive response at toxic levels rather than lethal levels is attractive due to greater biological relevance.

MATERIALS AND METHODS

Cell culture and treatments. NR8383 cells were maintained in culture according to the originator's recommendations (Helmke *et al.*, 1987). Cells from passages 10–25 were seeded at $4–6 \times 10^5$ cells/ml in modified Ham's F-12 medium (Life Technologies/GIBCO, Grand Island, NY) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO) and incubated at 37°C, 80% humidity, and 5% CO_2. Growth curves were established by combining the free-floating cells and the attached cells obtained following trypsinization with viability determined by trypan blue exclusion. A Model Z Coulter counter (Coulter Electronics, Inc., Hialeah, FL) was utilized for cell counts. Except as stated, chemicals were from Fisher Scientific, Inc. (Pittsburgh, PA).

To assess inhibition of cell proliferation by hydrogen peroxide, cells were treated 30 min postpassage with various concentrations of H_2O_2 and incubated undisturbed for 46 hr. Cell counts were made from five replicate flasks for each treatment dose. Control cultures were seeded from the same source and at the same time as H_2O_2-treated cells and were treated in an identical fashion, except that H_2O_2-free culture medium was added.

Mercaptosuccinate (MS), a known inhibitor of the cellular H_2O_2-neutralizing antioxidant enzyme glutathione peroxidase (Chaudiere *et al.*, 1984; Michiels and Remacle, 1988; Michiels *et al.*, 1994), was used to confirm that the responses observed were caused by increased intracellular levels of H_2O_2. MS was added to cultures in the same manner as the H_2O_2 in concentrations from 0 to 4500 μM.

RNA isolation and quantitation. NR8383 cells were exposed to H_2O_2, or MS, or to both H_2O_2 and MS at various concentrations in complete medium for 4 hr at 44–48 hr postpassage when these cells are known to be exponentially proliferating. Total RNA was isolated from cells by a modified method of Chomczynski and Sacchi (1987) using Trizol reagent (Life Technologies/GIBCO, Grand Island, NY). Nonadherent cells were pelleted at $450g_{max}$. Adherent cells were lysed and removed from the flask after incubation with Trizol reagent for 5 min and this cell lysate was added to the pelleted cells and incubated an additional 5 min. Addition of chloroform followed by centrifugation yielded an aqueous phase containing the RNA which was recovered by precipitation with isopropyl alcohol. RNA was resuspended in DEPC-treated H_2O and treated with DNase I using the MessageClean kit (GenHunter Corporation, Brookline, MA) which was followed by phenol:chloroform:isoamyl alcohol extraction and precipitation. The total RNA concentration was measured by the absorbance at 260 nm and the ratio of the absorbances at 260/280 nm was used to verify purity. The RNA concentration was adjusted to 1 μg/μl with DEPC–H_2O and the RNA was stored at −70°C.

RT-PCR. The analytical approach of Murphy *et al.* (1990) was used in this study. Briefly, 1 μg of total RNA was reverse-transcribed using the GeneAmp RNA PCR Kit (Perkin Elmer, Foster City, CA) murine leukemia virus reverse transcriptase, and random hexamers. The resulting cDNA was serially diluted 1.5-fold from 1:16 to 1:180,000 and amplified by PCR using intron-spanning primers designed with the Oligonucleotide Selector Program (Hillier and Green, 1991) for specificity to *GADD45* or *GADD153* and *β-ACTIN* as an internal control. The primers were synthesized by CRUACHEM, Inc. (Dulles, VA).

GADD45 primers:

Sense primer 5′tgcagatccatttcaccc (nt 230–247)
Anti-sense primer 5′tccatgtagcgactttcc (nt 479–462).
Primers were chosen using a rat *GADD45* mRNA sequence (GenBank No. RATGADD45X). Primers were chosen to span the intron between exons 3 and 4 and yielded a 250-bp product.

GADD153 primers:

Sense primer 5′cacctatatctcatcccc (nt 218–235)
Anti-sense primer 5′ccactttcctctcattctc (nt 501–483).
Primers were chosen using a rat *GADD153* mRNA sequence (GenBank No. RNU30186) and yielded a 284-bp product.

β-ACTIN primers:

Sense primer 5′tcaggtcatcactatcgg (nt 735–752)
Anti-sense primer 5′gatcttcatggtgctagg (nt 981–964).
Primers were chosen using rat *β-ACTIN* genomic sequence (GenBank No. RNAC01). Primers were chosen to span the intron between exons 3 and 4 and yielded a 247-bp product.

PCR reaction mixtures were composed of 4 μl of the respective dilution of cDNA template and 16 μl of the PCR master mix containing the appropriate primers for each gene. Amplification was performed for 30 cycles at 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min with a final extension time of 5 min at 72°C. Five microliters of loading buffer with dye was added to the 20-μl reaction mix and 20 μl of this mixture was loaded in a 2.5% agarose gel and electrophoresed at 100 V for 45 min in 1× Tris–borate–EDTA buffer. The gels were stained for 10 min with 0.5 μg/ml ethidium bromide,

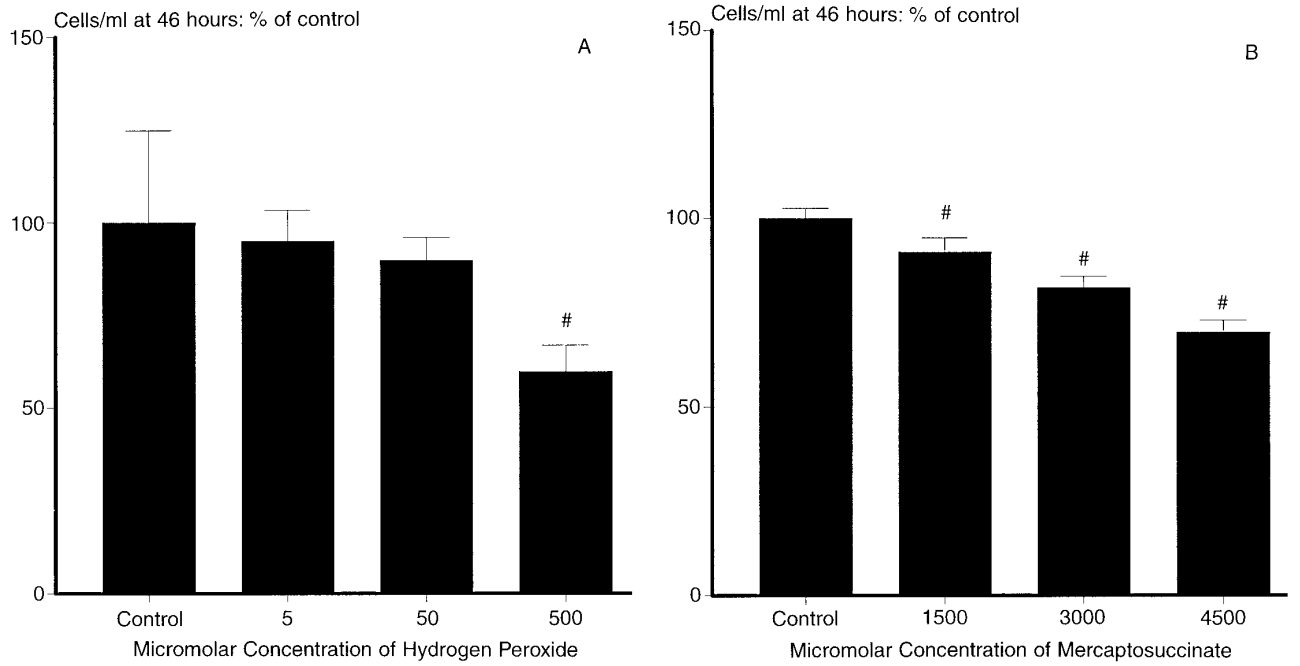

FIG. 1. Growth inhibition of NR8383 rat alveolar macrophage cells compared to that of controls at 48 hr. (A) Hydrogen peroxide at 5, 50, or 500 μM and (B) mercaptosuccinate at 1500, 3000, or 4500 μM. Hydrogen peroxide degrades approximately 80%/hr in such systems and therefore was deemed negligible after 3 hr. Significance (# $P < 0.01$ for H_2O_2 and $P < 0.05$ for mercaptosuccinate; $n = 5$) was determined by Student's t test comparison of the means (error bars, SEM).

destained for 30 min in water, and photographed using Polaroid 665 positive/negative film.

Analysis. The negatives were scanned with a LKB UltraScan laser densitometer with readings taken for three positions on each band. Peak area was calculated for each reading using GelScan XL software (Pharmacia LKB, Uppsala, Sweden). The results were entered into a SuperCalc spreadsheet (Computer Associates, Inc., San Jose, CA), the median value for each lane was recorded, and the results were plotted on a log–log scale against the original quantity of RNA present in the PCR reaction diluted from the reverse-transcription product (i.e., a 1:16 dilution of the original 1 μg of RNA that was in a 20-μl RT reaction is 3.125 ng/μl). The PCR reaction was considered to be exponential if 1.5-fold dilutions produced comparable reductions in band intensity. For each graph, a best-fit line was plotted based on the linear range of the data. The ratios of *GADD45*:*β-ACTIN* and *GADD153*:*β-ACTIN* were calculated directly from the plots for each sample. The ratio for each treatment was then divided by the ratio for the control to determine the magnitude of change as induction (Murphy *et al.*, 1990). Student's t test was used to determine levels of significance.

RESULTS

Effects of Oxidative Stress-Inducing Hydrogen Peroxide on Cell Proliferation

Over the period 1–3 days postpassage, NR8383 cultures expand logarithmically until densities surpass approximately 1.5×10^6 cells/ml when the replication rate slows. Treatment of NR8383 cells showed small but incremental cell growth inhibition in response to 5 and 50 μM H_2O_2 (Fig. 1A) by 46 hr compared to controls while cells treated with 500 μM H_2O_2 demonstrated significant inhibition ($P < 0.01$) to about 60% of the proliferation seen in control cultures; viability by trypan blue exclusion remained >97%.

Effects of Oxidative Stress-Inducing Mercaptosuccinate (MS) on Cell Proliferation

Treatment of NR8383 cells with MS, a specific inhibitor of GPx, had little effect up to 1000 μM (data not shown). In the range of 1500–4500 μM (Fig. 1B), significant inhibition ($P < 0.05$) of proliferation at 46 hr was noted with 4500 μM producing inhibition to about 70% of sham-treated control cultures.

Effects of Oxidative Stress Induced by H_2O_2 on GADD45 Expression Levels

RT-PCR of total RNA from treated and control cells was employed to measure the induction of *GADD45*. Empirical determination of the serial dilutions of cDNA required to achieve nondetection of ethidium bromide staining indicated that the *β-ACTIN* message was approximately 2 orders of magnitude more prevalent than *GADD45* in the isolated RNA. Thus, a 1:122 dilution of *GADD45* cDNA gave PCR reaction product with staining comparable to *β-ACTIN* at a 1:15,764-dilution. At these respective dilutions, the ratio of *GADD45*:*β-ACTIN* in control cells was 0.48 (SD ± 0.23)

TABLE 1
Ratios of Genes *GADD45*[a] and *GADD153* Normalized to *β*-ACTIN

	Control GADD:ACTIN		500 μM H_2O_2 treated GADD:ACTIN		Induction (treated/control)	
Exp. no.	*GADD45*	*GADD153*	*GADD45*	*GADD153*	*GADD45*	*GADD153*
1	0.20	0.04	1.0	0.14	5.0	3.5
2	0.32	0.13	0.88	0.22	2.8	1.7
3	0.33	0.13	1.7	0.30	5.2	2.3
4	0.58	0.18	2.7	0.54	4.7	3.0
5	0.90	0.52	4.2	1.0	4.7	1.9
6	0.55	0.35	4.2	0.86	7.6	2.5
Mean ± SD:	0.48 ± 0.23	0.23 ± 0.16	2.5 ± 1.4	0.50 ± 0.32	5.0 ± 1.4	2.5 ± 0.62

[a] GADD, growth arrest and DNA damage-inducible.

for six experiments (Table 1). In order to determine the reproducibility of such results within a single experiment, four replicate PCR amplifications of Experiment 4 cDNA were compared and yielded ratios of 0.40, 0.57, 0.82, and 0.52 (mean = 0.58, SD ± 0.15).

For six experiments, RT-PCR of total RNA from cells treated with 500 μM H_2O_2 showed a mean induction of 5-fold over controls when *GADD45* was normalized to *β-ACTIN* (Fig. 2). Cells treated with 10-fold serial dilutions below 500 μM H_2O_2 showed no difference in *GADD45* expression compared with that seen in controls (data not shown).

Effects of Oxidative Stress Induced by H_2O_2 on GADD153 Expression

To confirm that the induction of *GADD45* was a generalized response related to the H_2O_2 treatments, *GADD153*, known to be regulated coordinatively with *GADD45*, was quantitated by RT-PCR and also showed similar induction. The ratio of *GADD153*:*β-ACTIN* in control cells was 0.23 (SD ± 0.16) for the six experiments (Table 1) when using the same 1:122 dilution for comparison. *GADD153* was induced over controls at a level 50% that of *GADD45* for 500 μM H_2O_2 treatments, averaging a 2.5-fold increase (Table 1).

Effects of Oxidative Stress Induced by MS on GADD45 and GADD153 Expression

Figure 3A shows the response of *GADD45* to 4500 μM MS in NR8383 cells after 4 hr of incubation as determined by RT-PCR. *GADD45* levels are elevated 3-fold relative to controls at 4500 μM MS. To ensure that the response at 4500 μM MS was not unique to that dose, expression at 1500 and 3000 μM MS (which, as mentioned, also had yielded significant growth inhibition) were examined and found to be similarly elevated. The *GADD153* response to MS (Fig. 3B), while also elevated relative to controls over the range 1500–4500 μM MS, is generally less than *GADD45*. At 4500 μM MS, for example, *GADD153* is 2.7-fold.

Combined Effects of Oxidative Stress Induced by H_2O_2 and MS on GADD45 and on GADD153 Expression Levels

To examine the effects of combined inhibition of GPx with elevated H_2O_2 as might occur physiologically such as in nutritional deficiency compounded by inflammation, NR8383 cells were treated with 4500 μM MS and increasing levels of H_2O_2 at 5, 50, and 500 μM. This resulted in even greater induction of both *GADD45* and *GADD153* in a dose-dependent manner (Figs. 3C and 3D). *GADD45* again increased more than *GADD153* at each treatment level with *GADD45* reaching 10-fold induction and *GADD153* 7.5-fold over controls. Interestingly, even the lower doses of H_2O_2 at 5 and 50 μM, when added to cells pretreated with 4500 μM MS, resulted in levels of GADD gene induction greater than that which occurred with 500 μM H_2O_2 alone. Moreover, *β-ACTIN*, typically considered a housekeeping gene resistant to modulation by stressors, seemed to have lower expression at the combined treatment level of 4500 μM MS with 500 μM H_2O_2. RT-PCR of control samples evaluated at a dilution equivalent to 0.008 ng/μl (1:15,764 dilution) of RNA had a mean absorbance for *β-ACTIN* of 179 ($n = 6$) which was statistically indistinguishable from samples treated only with 500 μM H_2O_2 (mean = 177, $n = 6$). When these two data sets were combined and tested for significance against *β-ACTIN* expression level in samples treated with both 4500 μM MS and 500 μM H_2O_2 (mean = 86, $n = 5$), the result was positive at $P < 0.01$. Thus, it appears that the combined treatment actively reduces *β-ACTIN* mRNA levels. Since our results are normalized to the *β-ACTIN* levels between controls and treated samples, the reduction in *β-ACTIN* may account for the apparently greater *GADD45* and *GADD153* inductions in cells treated with both H_2O_2 and MS.

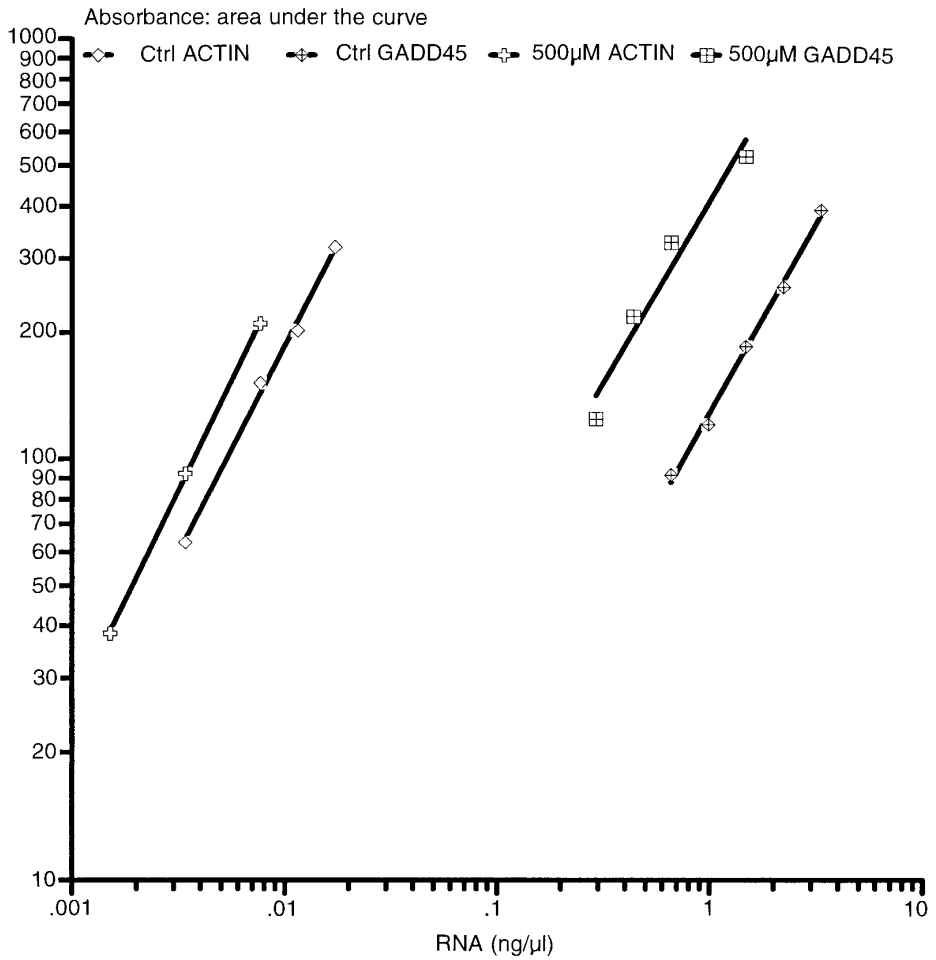

FIG. 2. Analysis of H_2O_2 effects on *GADD45* and *GADD153* gene expression levels normalized to *β-ACTIN*. NR8383 alveolar macrophages were treated with 500 μM H_2O_2 at the beginning of a 4-hr incubation. Utilization of *β-ACTIN* normalization for serially diluted PCR products over the exponential amplification range is shown. The *β-ACTIN* plots (left pair) are quantitatively similar, while there is a greater shift between the *GADD45* plot for the 500 μM H_2O_2 compared to the control. This shift, when computationally adjusted for the small *β-ACTIN* shift, demonstrates increased expression of *GADD45* gene relative to sham-treated controls.

DISCUSSION

The purpose of this investigation was to learn if alveolar macrophages expressed functional growth arrest and DNA damage-inducible genes and whether the genes would be modulated by oxidative stress. Hydrogen peroxide, which freely permeates the nuclear as well as other cellular membranes, does not react extensively with DNA but, under suitable conditions, forms hydroxyl radicals via a Fenton-like reaction involving transition metal ions and causes DNA damage in the forms of oxidized nucleotides and single strand breaks (Mello Filho and Meneghini, 1984). While H_2O_2 and hydroxyl radicals cause other cytotoxic effects and we cannot exclude the possibility of other effects of H_2O_2 in our system, recent work (Jornot *et al.*, 1991) employing scavengers and specific inhibitors has shown that hydroxyl radicals are responsible for DNA damage which can be expected to result in oxidative stress gene induction.

Oosting *et al.* (1990) and others have studied AM exposed to H_2O_2. The normal rat AM population is maintained at least in part by *in situ* proliferation (Shellito *et al.*, 1987) and ozone and asbestos, both capable of oxidative lung cell injury, have been associated with increases in local proliferation of AM cell populations (Mochitate *et al.*, 1989; Spurzem *et al.*, 1987). GADD gene expression in response to such DNA damage in other cell systems is readily observable in 4 hr (Fornace *et al.*, 1988) but has not been studied in AM. The genotoxic stress of DNA damage can occur during physiologic processes (e.g., inflammation, reperfusion injury, chemotherapy) or as a result of environmental pollutant exposure and can elicit a complex gene expression response in mammalian cells, including growth inhibition, enhanced

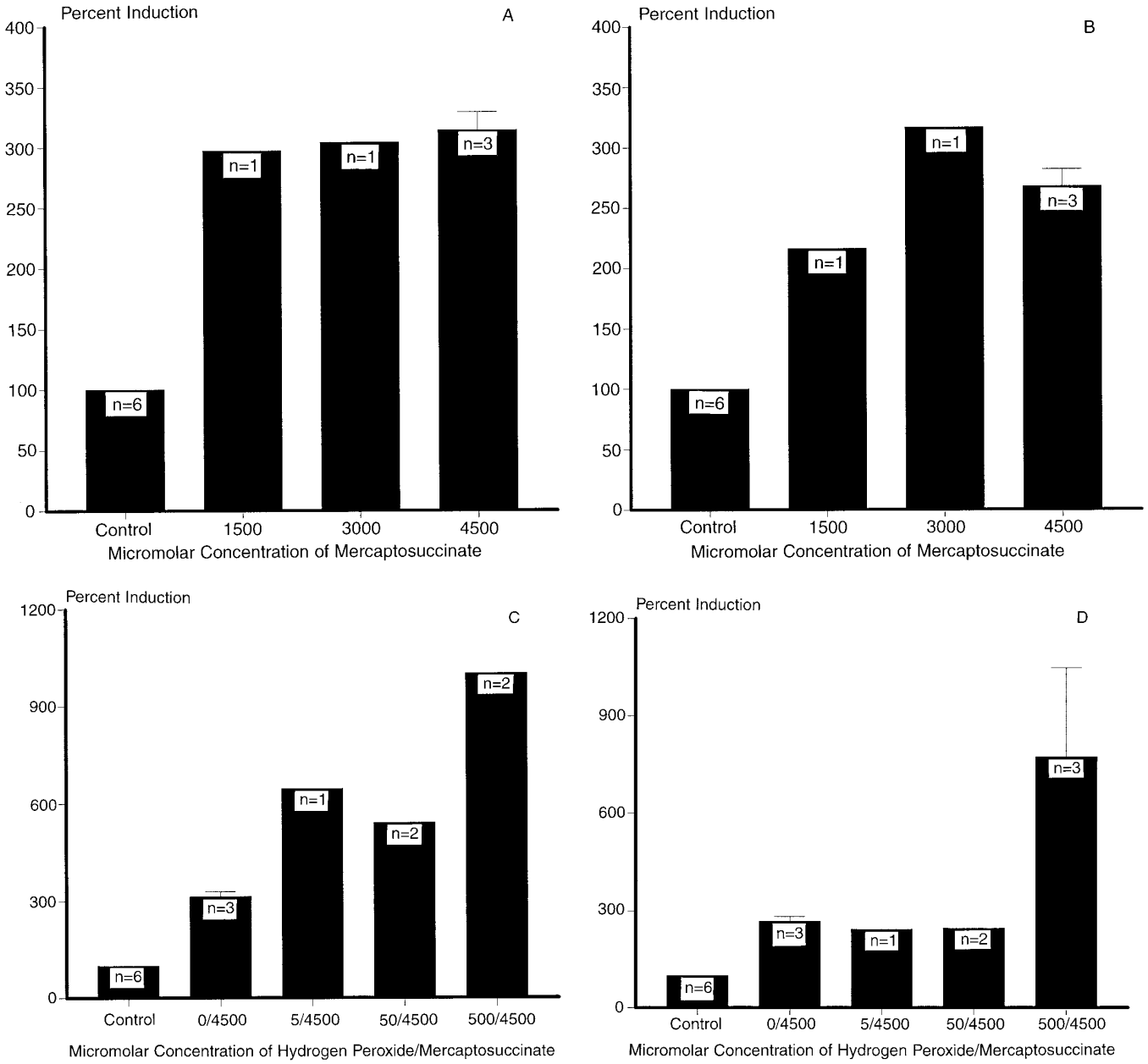

FIG. 3. Oxidative stress dose–response histograms normalized to *β-ACTIN* for NR8383 alveolar macrophage gene expression. Mercaptosuccinate, a specific inhibitor of the antioxidant enzyme glutathione peroxidase, elicits elevated levels of mRNA for genes known to respond to growth arrest and DNA damage, (A) *GADD45* and (B) *GADD153*. The induction of the two oxidative stress inducible genes (C) *GADD45* and (D) *GADD153* by mercaptosuccinate is augmented by exogenous H_2O_2 (error bars, SEM; by definition, controls are 100% and thus have no SEM).

protection or tolerance, and altered mutagenicity (Fornace, 1992). Our interest is in the ability to detect early response to DNA damage as a biomarker for environmental oxidative stress. It is reasonable to expect that materials capable of causing oxidative stress could inhibit normal AM functions and result in altered secretion of inflammatory mediators and growth factors.

Because it is difficult to separate the genotoxic effects of treatment agents from the growth-arresting effects, it is important to determine the influence of such agents on the growth and viability of cells (Crawford *et al.*, 1996). Although *GADD45* is induced by irradiation injury in nondividing normal tissue such as adult rat brain (Yoshida *et al.*, 1994), proliferating cultures offer the advantage of being sensitive to DNA damage as evidenced by cell growth inhibition, thereby providing a useful endpoint in response to geno-

toxic challenge. It was for this reason that H_2O_2 and MS treatment levels were selected which yielded comparable cell growth inhibition at which to compare GADD gene response. In the current work, *GADD45* expression increased 5-fold when cells were treated with 500 μM H_2O_2 and *GADD153* expression increased 2.5-fold, a level 50% that of *GADD45*. Since glutathione is a principal biomolecule by which cells protect against oxidative damage such as produced by H_2O_2, it is reasonable to expect that inhibition of GPx would produce similar GADD gene expression changes as seen with exogenous H_2O_2. When GPx was inhibited with 1500–4500 μM MS, *GADD45* expression was induced approximately 3-fold and *GADD153* approximately 2- to 2.5-fold. These results support a role for GPx in protecting cells from genotoxic damage. Combined exposures of H_2O_2 with MS resulted in even greater induction, demonstrating modulation of the antioxidant pathway protecting DNA from oxidative damage that can occur at sublethal toxic exposures. Indeed, elevated levels of GPx in stably transfected cells have been shown to protect against UVB-induced DNA strand breaks mediated in part by H_2O_2 (Ghosh *et al.,* 1993). Inhibition of DNA synthesis by glutathione depletion of T lymphocytes increased *HSP70* (heat shock protein 70) and *GADD153* mRNA levels (Walsh *et al.,* 1995). Further, it has been shown that overexpression of CuZn–superoxide dismutase produces an oxidative stress hypersensitivity which can be blocked by overexpression of GPx (Amstad *et al.,* 1994), thus underscoring the fact that small imbalances in physiological activities of antioxidants can alter oxidant-induced DNA damage and patterns of cell death.

We have shown that the toxic effects of H_2O_2, presumably through hydroxyl radical damage observed in the reduced proliferation of NR8383 alveolar macrophages, are associated with induction of *GADD45* and *GADD153.* The overall response protective of the genome lies in delaying cell cycle progression until DNA repair can be effected (Gately *et al.,* 1994; Luethy and Holbrook, 1992). The demonstrated oxidative induction of transcripts for *GADD153* in addition to *GADD45* supports the view that H_2O_2 in this model is stimulating a generalized cellular response to DNA damage. *GADD153* induction is known to be mediated in part by an AP-1 binding site in the promoter region (Guyton *et al.,* 1996), a widespread genomic regulatory domain for inducible genes.

Biogenic hydrogen peroxide concentrations have been measured *in vitro* and *in vivo* under a variety of conditions (reviewed in Burdon, 1995) and from these results it is clear that the actual intracellular effective concentration in these experiments is determined by multiple factors including microenvironment levels of enzymatic and nonenzymatic antioxidants at the site of molecular targets (Test and Weiss, 1984). Our results are consistent with reported inductions in other model systems such as 300 μM H_2O_2 treatments of fibroblasts which had no effect on cell viability but essentially obliterated DNA synthesis (Chen and Ames, 1994) and with the known increase in both protein level and transcriptional activity of p53 during oxidative stress, given that Gadd45 is a downstream effector for p53 (Zhan *et al.,* 1995). Gadd45 and $p21^{Waf1/Cip1}$ act as downstream effectors of both p53-dependent and p53-independent DNA damage-response pathways to cause growth arrest pending DNA repair (Zhan *et al.,* 1995; Kearsey *et al.,* 1995). Gadd45 and $p21^{Waf1/Cip1}$ may compete (Chen *et al.,* 1995; Kearsey *et al.,* 1995) for binding to PCNA (proliferating cell nuclear antigen) which binds cyclin D1 (Smith *et al.,* 1994). The Gadd45 homolog MyD118 and possibly other proteins also function in similar but distinct pathways controlling suppression of cellular growth and apoptosis (Vairapandi *et al.,* 1996). Taken together, these facts support the idea that the interactions help coordinate the cell cycle and DNA repair (Hall *et al.,* 1995). Also, it is likely that *GADD45* and *GADD153* synergistically contribute to growth inhibition as overexpression demonstrates an additive effect (Zhan *et al.,* 1994). The apparent additive contribution to GADD gene response by the combination of MS with H_2O_2 treatments in the current work suggests surplus capacity for cellular reaction to more severe DNA damage than seen with either MS or H_2O_2 alone.

GADD gene expression and induction have been reported in all normal mammalian cell types examined; however, this is the first description of the constitutive and induction levels for alveolar macrophages. While these results are expected, they extend the cytologic range to include phagocytes which are particularly endowed with functional abilities that both increase the risk of exposure to oxidative stress through phagocytosis of radical-mediating chemicals such as iron and silica from inhaled particles and generate elevated endogenous reactive oxidative species through the respiratory burst associated with phagocytosis. Human alveolar macrophages are more sensitive to noxious substances from airborne particulates than rat alveolar macrophages when phagocytotic ability and cell viability are quantitated (Hadnagy *et al.,* 1993). It will be valuable to learn how these gene expression levels change *in vivo* and whether they have a role in protecting against lung injury from ambient particles in normal rats and humans.

Various definitions have been applied to the concept of biomarkers for environmental exposures (Borm, 1994). At the core of this concept is a mechanistic connection between dose and relevant effects. Using known oxidative stress-responsive RNA induction appears valuable as a mechanistically based, biomarker-oriented approach for environmental particulate cytotoxicity. While the nature of antioxidant protection against genotoxicity remains complex, the approach of quantifying the cells' own genomic response to DNA damage seems efficacious for studying oxidative toxicity from causes such as environmental particulates.

One challenge facing researchers seeking to understand the cellular consequences of air pollution is the inherent complexity of particulates that may have both soluble and insoluble constituents. An *in vitro* system using phagocytes provides an opportunity to more closely mimic the *in vivo* situation where cells may be subjected to soluble and insoluble materials. RT-PCR provides a sensitive tool for visualizing early cellular response to genotoxic agents at concentrations below those that trigger apoptosis or produce necrosis. These molecular methods provide a means of moving beyond LD_{50}-style experimental approaches toward studies yielding mechanistic toxicological knowledge by determining the genomic response propagated by specific genes of interest. Also, new avenues to understanding species differences with reduced animal use may be possible. The application of techniques incorporating the growing understanding of biomolecular relationships and pathways offers a uniquely informative model approach for evaluating the genotoxicity of environmental particulates while providing new insights into the subcellular mechanisms of toxicity.

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